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FIELD EVALUATION OF AN ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR *PLASMODIUM FALCIPARUM* SPOROZOITE DETECTION IN ANOPHELINE MOSQUITOES FROM KENYA

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Abstract. An enzyme-linked immunosorbent assay (ELISA) using a monoclonal antibody that recognizes a repetitive epitope on the circumsporozoite protein of *Plasmodium falciparum* was used in Kenya to assess malaria infections in *Anopheles gambiae* s.l. and *An. funestus*. The ELISA confirmed that 88% of 44 sporozoite-positive gland dissections were *P. falciparum*. The ELISA infection rate of 18.6% (n = 736) for individually tested mosquitoes for both species was significantly higher than the 10.4% (n = 537) salivary gland sporozoite rate determined by dissection. This difference was due to ELISA detection of medium and large sized oocysts on the midguts of infected mosquitoes which did not contain salivary gland sporozoites. From a series of 379 *Anopheles* that were cut at the thorax, ELISA tests on "head" and "body" portions showed that 29.5% of 95 positive mosquitoes contained circumsporozoite antigen in the body portion in the absence of salivary gland infections. This field evaluation demonstrates that the ELISA can most accurately be used to estimate sporozoite rates by cutting mosquitoes at the thorax and testing anterior portions.

Malaria transmission is ultimately dependent upon the sporozoite rate: the proportion of female anopheline mosquitoes with sporozoites in their salivary glands. Many factors determine sporozoite rates in natural mosquito populations, including mosquito susceptibility, population densities, feeding habits, longevity, duration of the extrinsic malaria cycle, and other host-vector parameters.¹ The entomological inoculation rate, defined as the product of the sporozoite rate and the human-biting rate, is the most valuable parameter for quantifying malaria transmission and predicting malaria epidemics.^{2,3} Assessment of these parameters will become especially important as sites are investigated for malaria vaccine trials.⁴⁻⁶

In addition to classical mosquito dissection for determining malaria sporozoite rates, two techniques have been developed which utilize mono-

clonal antibodies specific for the circumsporozoite (CS) protein. The immunoradiometric assay (IRMA) was developed using monoclonal antibodies against 4 malaria species.⁷ The IRMA was sensitive enough to detect fewer than 100 sporozoites, and for the first time it was possible to differentiate *Plasmodium* species in the mosquito. The ELISA was later adapted for the same purpose and proved equally reliable.^{8,9} These techniques offer several theoretical advantages over dissection: 1) sporozoites can be identified by species; 2) specimens can be held in storage (dry or frozen) before processing; and 3) numbers of sporozoites can be estimated. Dissection techniques require careful microscopic examination of freshly-caught mosquitoes by trained personnel, under sometimes difficult field conditions. We have pursued development of an ELISA sporozoite detection system because it does not require radioactive reagents or a gamma counter for analysis. Furthermore, the reagents used are stable for long periods of time and tests in 96-

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well microtiter plates can be read either visually or quantified with a spectrophotometer.

Immunological techniques for sporozoite detection in mosquitoes will improve capabilities of malaria field programs. The IRMA proved highly sensitive for detecting *Plasmodium falciparum* infections in *Anopheles gambiae* s.l. mosquitoes in The Gambia and West Africa,¹⁰ and recently, *P. falciparum* and *P. vivax* assays were tested in Papua New Guinea¹¹ and for mosquitoes from the Thai-Kampuchea border area.¹² We report here the results of the first field evaluation of the ELISA technique in Africa.

MATERIALS AND METHODS

Study area

The study was conducted near Kisumu in western Kenya. This area is holoendemic for *P. falciparum*, though *P. malariae* and *P. ovale* infections are also seen. Mosquitoes were collected around the villages of Saradidi (55 km west of Kisumu) and Tiengre (10 km west of Kisumu). Both sites were studied by the World Health Organization (1972 to 1976) as unsprayed comparison areas for the evaluation of fenitrothion as a residual insecticide.¹³⁻¹⁶ These studies described malaria transmission by the *An. gambiae* Giles complex and *An. funestus* Giles. During the present study, no mosquito control measures were in effect.

Anopheline collections

Mosquitoes were collected from September to October 1985, before the short rainy season, from a 3 km² area around each site, with collections primarily at the Saradidi site. Daytime collections inside houses were made by aspirating anopheline mosquitoes from the walls, ceilings, and other objects. Mosquitoes from each house were placed in screened pint cartons. Specimens were transported to the Division of Vector-Borne Diseases laboratory in Kisumu. Most were processed within 24 hr but some were held up to 72 hr by providing mosquitoes with 5% sugar solution on cotton. Mosquitoes were sorted and identified to species based on taxonomic criteria. Only *An. gambiae* s.l. and *An. funestus* were analyzed in this study. The *An. gambiae* complex in this area consists of *An. gambiae* and *An. ara-*

biensis but we did not attempt to separate these 2 species by chromosomal or electrophoretic analysis.¹³⁻¹⁶ For each collection, specimens from each house were randomly divided into two groups, one for dissection and the other for ELISA.

Dissections

Dissections involved first examining the ovaries of each mosquito for parity.¹⁷ Midguts and salivary glands of parous mosquitoes were then examined for oocysts and sporozoites, respectively. Oocysts were counted, graded according to relative size, and differentiated oocysts and free sporozoites were noted. Salivary gland sporozoite infections were graded either 1+ (1-10 sporozoites), 2+ (11-100 sporozoites), 3+ (101-1,000 sporozoites), or 4+ (>1,000 sporozoites).

ELISA method

The basic ELISA method for sporozoite detection was developed by Burkot et al.,⁹ based on the immunoradiometric assay of Zavala et al.⁷ The assay used in this study was 10 times more sensitive than the original *P. falciparum* ELISA and was based on results from the comparative testing of 10 *P. falciparum* sporozoite monoclonal antibodies for development of standardized ELISAs with improved sensitivity.¹⁸ The *P. falciparum* ELISA employed the 2A10 monoclonal antibody¹⁹ which recognizes the CS tetrapeptide, Asn-Ala-Asn-Pro.⁴

After identification, mosquitoes were placed in 0.5 ml capped polypropylene vials by species and house number, and frozen in liquid nitrogen. Specimens were transported within 1 week to the Kenya Medical Research Institute (KMRI) in Nairobi, where they were held at -20°C. Mosquitoes were ground in 50 µl blocking buffer (BB) (for 1 liter: 10 g BSA; 5.0 g Casein; 0.1 g Thimerosal; 0.01 g Phenol red; 1,000 ml phosphate buffered saline (PBS) pH 7.4) with Nonidet P-40 (NP40) (5 µl NP40/1 ml BB) using glass grinders. Then 200 µl BB was added to bring the final volume to 250 µl per mosquito.

Mosquitoes were tested individually by the ELISA method in 96-well soft microtiter plates. Fifty µl of monoclonal antibody (0.1 µg/well) were put in each well and incubated overnight at room temperature. After aspirating the monoclonal

TABLE 1

Salivary gland and gut infections detected by dissection and tested by ELISA to determine *Plasmodium falciparum* reactions

Dissection		Number tested	Positive with <i>P. falciparum</i>		Mean absorbance for positive reactions
			Number	%	
Salivary gland*					
Sporozoite grade:	1+	7	5	71.4	1.52
	2+	16	15	93.8	1.61
	3+	8	6	75.0	1.83
	4+	12	12	100.0	1.63
Total		44†	39	88.6	1.60
Midgut					
Oocyst size:					
	Small	7	0	0	—
	Medium	10	6	60.0	1.34
	Large	9	5	55.5	1.56
	Mixed sizes	3	2	66.7	1.09
Free sporozoites		1	1	100.0	0.84
Total		30	14	46.7	1.34

* Salivary gland preparations also contained sporozoites from the glands and other mosquito tissue to maximize the probability of detecting *P. falciparum* circumsporozoite antigen.

† Includes one ungraded infection that showed a positive reaction.

antibody solution, and incubating plates filled with BB for 1 hr, plates were aspirated and 50 μ l of each homogenized mosquito was added per well. After 2 hr incubation, wells were washed twice with PBS-Tween 20 (0.5 ml Tween 20/1 liter PBS, pH 7.4). Horseradish peroxidase (HRP)-conjugated monoclonal antibody (0.1 μ g/50 μ l BB/well) was then added to each well and after 1 hr incubation, plates were washed 3 times with PBS-Tween 20. Finally, 100 μ l of peroxidase substrate (Kirkegaard and Perry) was added per well and plates were read using an ELISA reader (414 nm) at 15 and 30 min.

Positive and negative controls were run on each plate. The positive control consisted of recombinant *P. falciparum* CS protein produced in *Escherichia coli*.¹¹ Uninfected negative control mosquitoes consisted of field collected male *An. gambiae* s.l. and *An. funestus*. Mosquitoes were considered infected if ELISA absorbance values (at 30 min) exceeded the mean plus 3 SD of 5 control mosquitoes on the same plate.

ELISA testing of dissection-positive mosquitoes

A series of dissection-positive mosquitoes was tested by ELISA. For salivary glands containing sporozoites the coverslip was removed from the slide and a saline rinse containing sporozoites,

glands, and mosquito debris was transferred into a 0.5-ml polypropylene vial using a 20- μ l capillary tube. This was held at -20°C with 100 μ l BB containing NP40 added prior to testing. For midguts containing oocysts, the coverslip was removed and the midgut (with minimal mosquito debris) was placed in a vial. This was handled and tested the same as gland preparations. Because midgut dissections were done in saline plus mercurochrome, a series of controls was run by ELISA to determine the effects of mercurochrome. Using known infected and uninfected mosquitoes, the addition of mercurochrome and/or saline, in concentrations used for dissections, had no effect on ELISA absorbance values.

ELISA testing of divided mosquitoes

The ELISA was also used to test mosquitoes which were divided into 2 parts by cutting them transversely at the thorax between the first and third pairs of legs (cuts were done with a scalpel blade while observing specimens at $\times 10$ – 20 with a dissection microscope). For these mosquitoes, the "head" and "body" portions were prepared and tested as described for the whole mosquitoes (including diluting each aliquot to a final volume of 250 μ l BB). This test series included 276 mosquitoes from Saradidi and 103 from Tiengre.

TABLE 2

Malaria infection rates determined by dissection and ELISA for *An. gambiae* s.l. and *An. funestus* collected inside houses in Saradidi from 13 September to 9 October 1985

Species	Dissection					ELISA		
	Number		Infection rate (%)			Number		(%)
	Dissected	Infected	Gut	Salivary glands	Overall	Tested	Positive	
<i>An. gambiae</i>	156	31	12.2	10.9	19.9	176	36	20.4
<i>An. funestus</i>	381	97	20.7	10.2	25.5	560	101	18.0
Total	537	128	18.2	10.4	23.8	736	137	18.6

RESULTS

ELISA testing of dissection-positive mosquitoes

A series of salivary gland and midgut infections detected by dissection were tested by ELISA (Table 1). ELISA tests were positive for 39/44 (88.6%) salivary gland infections and 14/30 (46.7%) midgut infections. The ELISA was sensitive enough to give positive readings for each sporozoite grade class and mean absorbance values in each class exceeded 1.50 on a scale of 0 to 2.00. Mosquitoes with medium, large, mixed-size oocysts, and free sporozoites in the midgut were positive with optical density values generally >1.00. Mosquitoes with midgut infections containing only small oocysts were not positive by ELISA.

Dissection vs. ELISA infection rates

Malaria infection rates were determined both by dissection and ELISA (Table 2). Since a range of malaria developmental stages can be determined by dissection, infection status is presented according to midgut (oocysts), salivary glands (sporozoites), and overall infection rates (oocysts and/or sporozoites). ELISA rates of 20.4% for *An. gambiae* s.l. and 18% for *An. funestus* were nearly twice as high as salivary gland sporozoite rates (10.9% and 10.2%, respectively). For both species, ELISA infection rates were more similar to overall dissection rates than to salivary gland rates.

Figure 1 illustrates the cumulative malaria infection rates determined by dissection and ELISA for 11 indoor collections in Saradidi from 13 September to 9 October 1985. These combined results for the 2 vector species show that the differences between infectivity rates determined by ELISA or salivary gland dissection were con-

sistent over a 1-month period. The ELISA rate most closely paralleled the overall dissection-positive rate minus "small oocysts." For this comparison, the cumulative rate determinations differed by only 2% at the end of the 1-month period.

ELISA testing of divided mosquitoes

A total of 379 anophelines were cut transversely at the thorax and ELISA tests were run in parallel on "head or body" portions (Table 3). Of 95 positive specimens, 70.5% were positive for the head portion (head: body ++ or +-) and 29.5% showed positive reactions for the body portion in the absence of head infections. It is noteworthy that 40% of *An. gambiae* s.l. compared to 21.8% of *An. funestus* showed reactions in the body but not in the head portion.

Oocyst infections

A frequency histogram of the number of oocysts per midgut-infected *An. gambiae* and *An. funestus* shows that most midgut infections contained 1 or 2 oocysts (Fig. 2). Both species exhibited similar patterns and only 11% (2/18) of *An. gambiae* (s.l.) and 9% (7/76) of *An. funestus* contained >10 oocysts. From dissection estimates of oocyst relative size, *An. gambiae* (n = 18) infections included 22% small, 28% medium, 33% large, 6% mixed-sized, and 11% not determined; *An. funestus* (n = 75) had 24% small, 52% medium, 12% large, 11% mixed, and 1% not determined. Differentiated and/or free sporozoites were observed in midgut dissections to determine what proportion of each species contained developing sporozoites in the midgut. Rates for the 2 combined categories were 38.9% (7/18) for *An. gambiae* and 18.7% (14/75) for *An. funestus*.

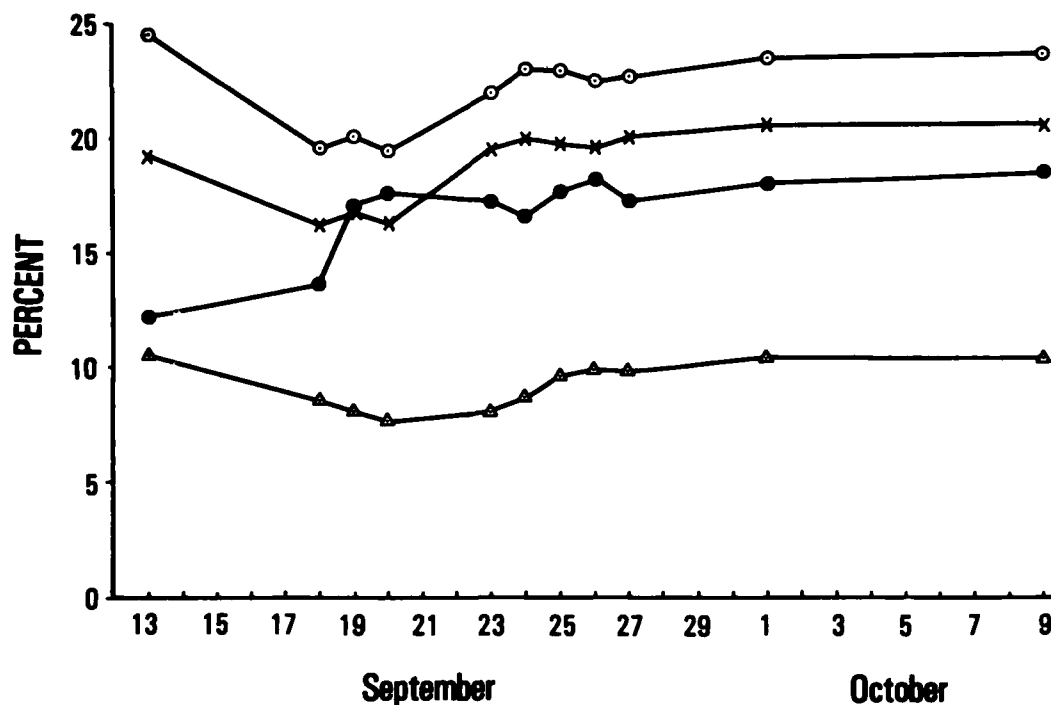


FIGURE 1. Cumulative measures of infection rates for *An. gambiae* s.l. and *An. funestus* (combined) determined from collections at Saradidi where specimens were dissected (Δ , salivary gland sporozoite rate; \circ , overall infection rate; X, overall infection rate minus small oocysts) and tested by ELISA (\bullet). This comparison is based on 1,273 mosquitoes (537 dissected and 736 tested by ELISA) from 11 daytime resting collections inside houses.

Frequency distribution of ELISA absorbance values

Positive reactions for *P. falciparum* circumsporozoite antigen were easily discriminated from negative control, uninfected mosquitoes. Cut-off absorbance values for positives were usually around 0.10, based on the mean and 3 SD for 5 uninfected mosquitoes on each microtiter plate. Species frequency distributions for infection intensity is compared for whole body and "head" ELISA tests (Fig. 3). Over 90% of the reactions were at least twice as high as the cut-off absor-

bance values. For whole body tests, 46.5% (27/58) of *An. gambiae* s.l. and 53.8% (64/119) of *An. funestus* had ELISA values > 2.00 . Similarly, for tests with head portions, values > 2.00 were observed for 62.5% (15/24) *An. gambiae* s.l. and 41.9% (18/43) *An. funestus*.

DISCUSSION

The ELISA offers new possibilities for identifying sporozoite infections according to malaria species. Our tests on dissected specimens showed

TABLE 3
Positive ELISA reactions for 99 *An. gambiae* s.l. and 280 *An. funestus* cut transversely at the thorax (2 tests/mosquito) to determine reactivity in the head vs. body

Type of infection		<i>An. gambiae</i>		<i>An. funestus</i>		Total	
Head	Body	No.	%	No.	%	No.	%
+	+	22	55.0	37	67.3	59	62.1
+	-	2	5.0	6	10.9	8	8.4
-	+	16	40.0	12	21.8	28	29.5
Total		40		55		95	

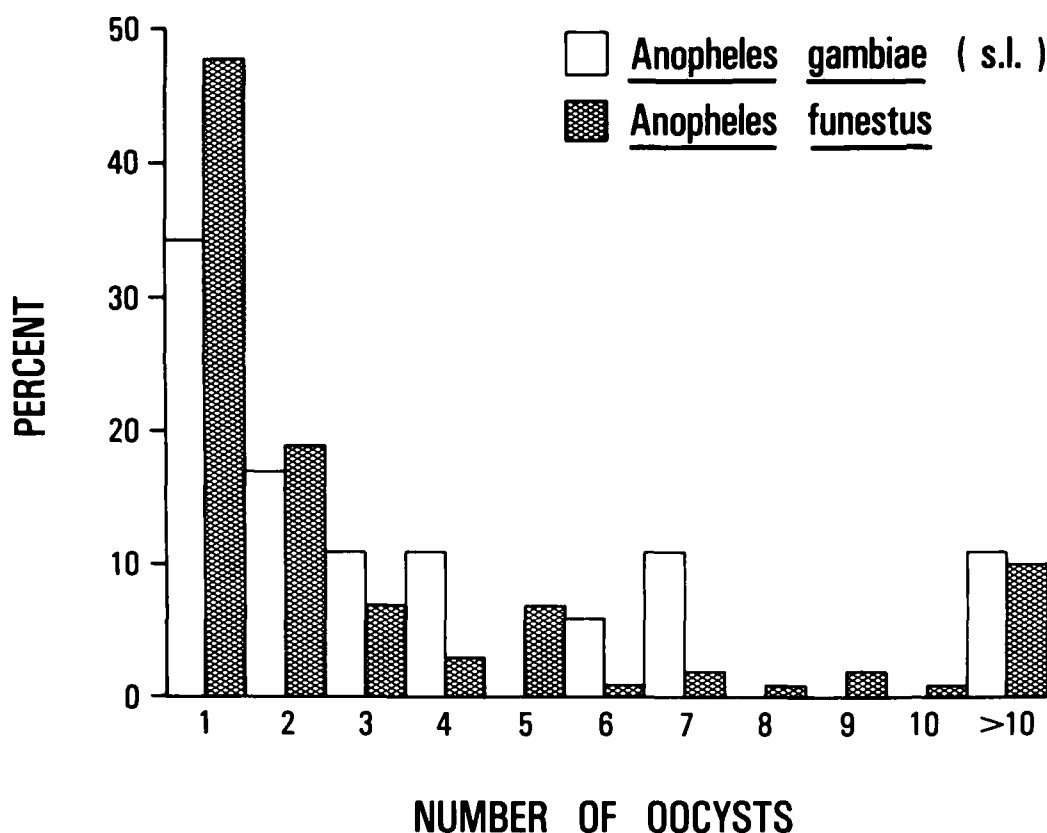


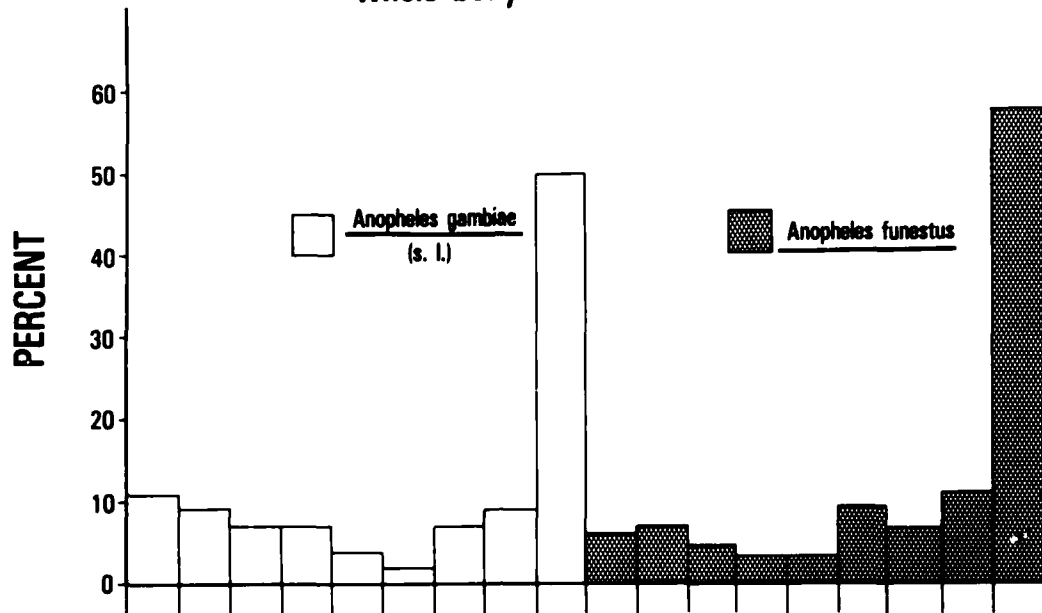
FIGURE 2. Frequency distribution of the number of oocysts per midgut-infected *An. gambiae* s.l. (n = 18) and *An. funestus* (n = 76).

that 88% of the salivary gland infections were *P. falciparum*. It is likely that the ELISA-negative infections were either *P. malariae* or *P. ovale*. This would correlate well with the observed species incidence in the human population (1980–1983) of 92% *P. falciparum*, 6% *P. malariae*, and 2% *P. ovale* (A. D. Bradling-Bennett, CDC, personal communication). In areas such as this with more than one malaria species, there would be obvious advantages to using monoclonal antibody based assays capable of detecting and differentiating all species present. In addition to the *P. falciparum* ELISA, a similar *P. vivax* test has been developed.²⁰ When tests are available for all human malarias, it will be possible to examine transmission of each species.

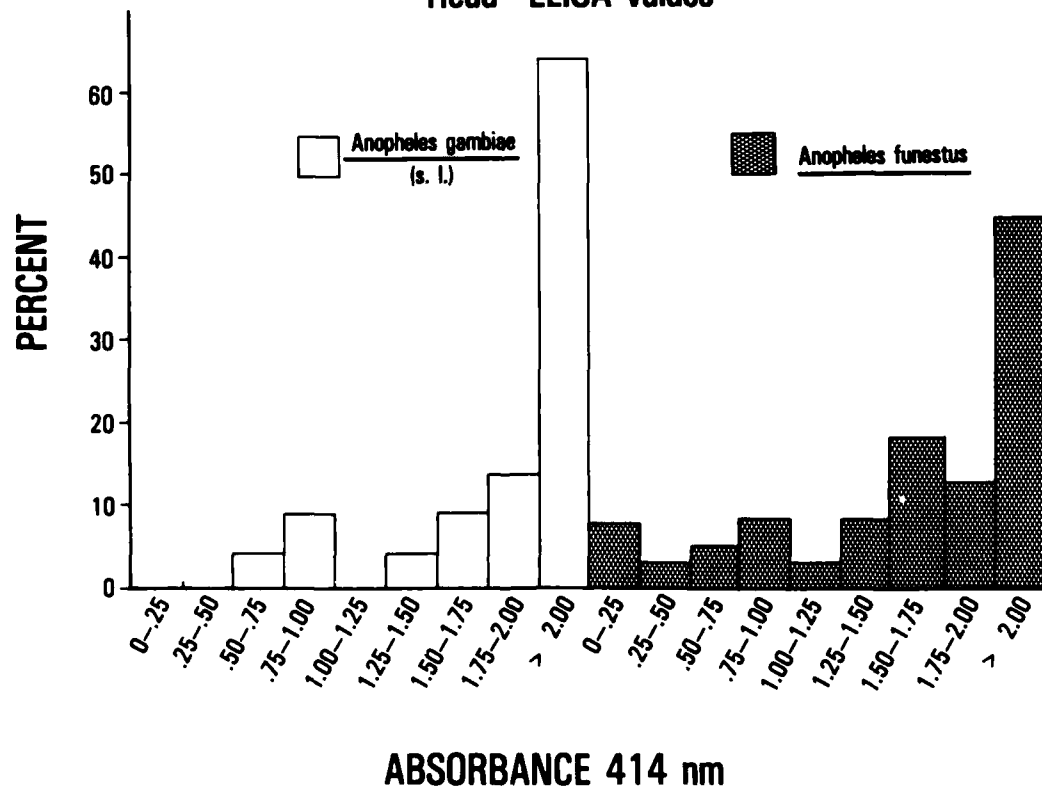
An important consideration is that ELISA infection rates for whole mosquitoes are not analogous to sporozoite rates determined by dissection. Since the ELISA detected medium and large sized oocysts in the midgut of mosquitoes that did not have sporozoites in the salivary glands, the test greatly overestimated sporozoite rates. The ELISA infection rate most closely paralleled the overall dissection infection rate minus those mosquitoes with small oocysts. The tests probably detected infections at least 4 days before sporozoites reached the salivary glands. This degree of sensitivity observed for the ELISA was not expected since earlier field studies with the IRMA, using the same monoclonal antibody, suggested that *P. falciparum* sporozoite antigens

FIGURE 3. Frequency distribution of ELISA absorbance values for positive mosquitoes. Top figure illustrates values for "whole body" tests of 58 *An. gambiae* s.l. and 119 *An. funestus*. Bottom figure shows values of "head" portions from mosquitoes cut at the thorax (24 *An. gambiae* s.l. and 43 *An. funestus*).

"Whole body" ELISA values



"Head" ELISA values



were detectable in the midgut only slightly before the appearance of sporozoites in the salivary glands.¹⁰

Given the excellent sensitivity of the ELISA, different strategies are available for using this assay to estimate sporozoite rates. One approach would be to determine a "correction factor" from subsamples of divided specimens (Table 3). However, this would require an assumption that the correction factor would remain stable for vector populations. Dissection results have consistently shown that oocyst infection rates are not constant in vector populations. Furthermore, correction factors would have to be employed for each vector species since our ELISA tests on divided specimens indicated that a higher proportion of *An. gambiae* contained sporozoite antigen in the midgut than *An. funestus*. A more reliable approach, and one that we are now using in our Kenyan field sites, involves cutting every specimen at the thorax and testing anterior portions by ELISA. This yields ELISA infection rates that are comparable to sporozoite rates determined by dissection (P. V. Perkins, personal communication). We are also testing whether any cut, dissected specimens without sporozoites in the salivary glands could possibly contain sporozoites in the thoracic hemocoel.

Observations on ELISA detection of midgut oocyst infections in mosquitoes without sporozoites in the salivary glands has important implications for vector incrimination. Positive ELISA results on potential vectors does not prove vector status, since specimens may not have sporozoites in the salivary glands. Some mosquito species develop heavy malaria infections but are not vectors since sporozoites do not invade salivary glands.²¹ Thus the ELISA on whole mosquitoes cannot replace observations on dissected specimens for vector incrimination.

In Africa, *An. gambiae* and *An. funestus* usually contain a small number of oocysts, however, resulting sporozoite levels usually range from 2,000–4,000; fewer than 5% contain <500 sporozoites.²² Collins et al. showed that the IRMA was sensitive enough to detect mosquito infections resulting from 1 oocyst.¹⁰ Our dissections also showed that most midgut-infected mosquitoes contained 1 or 2 oocysts. CS antigen was easily detected by ELISA in both whole and cut *An. gambiae* s.l. and *An. funestus* (Fig. 3). The threshold of sensitivity of the ELISA is around

125 sporozoites per mosquito (25 sporozoites per 50 μ l; $\frac{1}{5}$ of a mosquito sample originally diluted to 250 μ l).¹⁸ Recently, techniques for sporozoite quantification by ELISA have been developed; absorbance values of samples are compared to standard curves of recombinant *Plasmodium* protein and counted sporozoites.^{11,12} Using the technique described by Wirtz et al.,¹¹ it was estimated that Kenyan mosquito samples with an absorbance value of 2.00 contained at least 1,500 sporozoites. Since absorbance values for about half of the infected mosquitoes exceeded 2.00, further tests on diluted samples will be required to estimate the number of sporozoites per mosquito. Wirtz et al.¹¹ have estimated that vectors in Papua New Guinea have geometric means of 929 *P. falciparum* and 460 *P. vivax* sporozoites, and Baker et al.¹² have suggested that primary vectors may have heavier sporozoite loads than secondary vectors. Since the number of sporozoites inoculated into the host influences the probability of acquiring an infection, many questions regarding sporozoite levels in vectors can now be answered using ELISA techniques.

The ELISA has many potential uses in malaria field studies, provided it is used accurately with an awareness of how ELISA results can be interpreted. One potential misconception is that ELISA tests on whole mosquitoes can totally replace dissections for determining sporozoite rates in vectors. Because the ELISA detects a large proportion of infected mosquitoes before sporozoites reach the salivary glands, this assay overestimates the actual sporozoite rate. If study objectives demand the precise determination of the sporozoite rate, the ELISA can best be employed by cutting specimens at the thorax and testing "head" portions. Valuable information regarding parity, age-grading, and oocyst infections are also lost if programs rely solely on the ELISA. Another misconception is that considerable time is saved with ELISA. This is not necessarily the case for individual mosquitoes but would be true for pools of mosquitoes (appropriate in areas with infection rates <1%). The *P. falciparum* ELISA employing the 2A10 has wide applicability because this monoclonal antibody reacts with sporozoites throughout the world,²³ a fact not surprising due to known conservation of the repeat region of the CS gene of *P. falciparum* in a wide variety of geographic isolates.²⁴

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The views of the authors do not purport to reflect those of the U.S. Army or the U.S. Department of Defense. The use of trade names is for identification only and does not imply endorsement by the U.S. Army or the U.S. Department of Defense.

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